Supplementary methods

Animal preparation

Experiments were carried out on Female Sprague-Dawley rats (2-3 mo old, 220 to 250 g, Hilltop Laboratory Animal, Scottdale, PA). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures and animal use were approved by the Animal Care and Use Committee at West Virginia University (ACUC# 06-0907). Female rats were used because the mesenteric vascular bed is more developed than in male rats, facilitating the selection of suitable venular microvessels for cannulation. Subcutaneous pentobarbital sodium was used for anesthesia, with an initial dosage of 65 $mg.kg^{-1}$ body wt and an additional 3 mg dose administered as needed. A midline surgical incision (1.5 to 2 cm) was made in the abdominal wall. The mesentery was gently moved out of the abdominal cavity and spread over a pillar for Lp measurements or over a glass coverslip attached to an animal tray for endothelial $[Ca^{2+}]_i$ and nitric oxide measurements. The upper surface of the mesentery was continuously superfused with mammalian Ringer solution at 37° C. Venular microvessels free of firmly attached leukocytes and with brisk blood flow were selected for the experiments. The diameter of the selected vessels ranged between 40 and 50 μm. Each experiment was carried out on a single microvessel from each animal.

Measurement of L_p in single perfused rat mesenteric microvessels

All measurements were based on the modified Landis technique, which measures the volume flux of water across the microvessel wall. The assumptions and limitations of the original method and its application to mammalian microvessels have been evaluated in details elsewhere.[1, 2] Briefly, a single venular microvessel was cannulated with a glass micropipette and perfused with

albumin-Ringer solution (control) containing 1% (vol/vol) hamster red blood cells as markers. A hydrostatic pressure (range 50–70 cmH_2O) controlled by a water manometer was applied through the micropipette to the microvessel lumen. A charge-coupled device camera was connected to the microscope, and video was recorded from a segment of the perfused microvessel (400 μm long) throughout the experiment for data analysis. The initial water flow per unit area of microvessel wall [$(J_v/S)_0$, where J_v is water flux and S is unit area of the microvessel wall] was calculated from the velocity of the marker cell after vessel occlusion, the vessel radius, and the distance between the marker cell and the occlusion site. Microvessel L_p was calculated as the slope of the relation between $(Jv/S)_0$ and the pressure difference across the microvessel wall. In each experiment, the baseline Lp and the Lp after application of testing solution were measured in the same vessel. This allowed the test agent-induced changes in Lp to be compared with its own control. The changes in Lp were expressed as the ratio of test agent-induced Lp to control Lp (Lp_{test} / $Lp_{control}$). The mean L_p for each perfusate was calculated from all occlusions during that perfusion period, if L_p is relatively constant in the entire time course. Otherwise, L_p is reported as the means of peak and sustained values, if a transient increase in L_p is observed.

Measurements of endothelial $[Ca^{2+}]_i$

Endothelial $[Ca^{2+}]_i$ was measured in individually perfused microvessels using the fluorescent Ca^{2+} indicator fura 2-AM. Experiments were performed on a Nikon Diaphod 300 microscope equipped with a Nikon photometry system including photometer head and finder (P101), computer-controlled shutter, and filter changer (Lambda 10-2; Sutter Instrument; Novato, CA). A rectangular variable diaphragm located in the photometer finder determined the size of the measuring window through which the fluorescence intensity (FI) was collected. In each experiment, a venular microvessel in rat mesentery was cannulated and perfused first with

albumin-Ringer solution that contained 10 μM fura 2-AM for 45 min. The vessel was then recannulated and perfused with albumin-Ringer solution for 10 min to remove fura 2-AM from the vessel lumen. A segment of fura 2-AM-loaded vessel at least 100 μm away from the cannulation site was then positioned within the field of view of the measuring window. The size of the window was adjusted to ~150 x 50 μm, which covered ~50 endothelial cells forming the vessel wall. A Nikon Fluor lens (X20, numerical aperture, 0.75) was used to collect FI values. The excitation wavelengths for fura 2-AM were selected by two narrow-band interference filters (340 ± 5 and 380 ± 5 nm; Oriel), and the emission was separated with a dichroic mirror (DM400) and a wide-band interference filter (500 ± 35 nm; Oriel). The excitation wavelength alternated between 340 and 380 nm, and corresponding FI values (FI_{340} and FI_{380}, respectively) were collected with a 0.25-s exposure at each wavelength. At the end of the experiment, the microvessel was superfused with a modified Ringer solution (5 mM Mn^{2+} without Ca^{2+}) while perfused with the same solution that contained ionomycin (10 μM) to bleach the Ca^{2+}-sensitive form of fura 2. The background FI due to unconverted fura 2-AM and other Ca^{2+}-insensitive forms of fura 2 were subtracted from FI_{340} and FI_{380} values. The ratios of the two FI values were converted to Ca^{2+} concentrations using an in vitro calibration curve.[3]

Fluorescence imaging of endothelial nitric oxide production

Endothelial nitric oxide levels were visualized and quantified at cellular levels in individually perfused microvessels using a fluorescence imaging system and 4,5-diaminofluorescein diacetate (DAF-2 DA), a membrane-permeable fluorescent indicator for nitric oxide. The experimental rigs were the same as that used for Ca^{2+} measurements, except that a 12-bit digital, cooled, charge-couple device camera (ORCA; Hamamatsu) was used for image acquisition. The excitation wavelength for DAF-2 DA was selected by an interference filter (480/40 nm), and

emission was separated by a dichroic mirror (505 nm) and a band-pass barrier (535/50 nm). In each experiment, a venular microvessel was cannulated and perfused first with albumin-Ringer solution that contained DAF-2 DA (5 μM) for 45 min. After loading, the dye was washed out from the vessel lumen with albumin-Ringer perfusate for 10 min before acquiring control images. Under control conditions, individual endothelial cells forming the vessel wall were slightly visible. An objective (X20, 0.75 numerical aperture) was focused on a group of endothelial cells that were at the same focal plane of the microvessel wall. In each experiment, images under control conditions and after exposure to testing agents were acquired from the same group of endothelial cells in each vessel using identical instrument settings. In case where vessels moved, the focus was readjusted to the maximum FI_{DAF} level for that group of endothelial cells. To minimize photobleaching, a neutral-density filter (0.5) was positioned in front of the excitation filter, and the exposure time was 80 ms at 30-s intervals. At the end of each experiment, the nitric oxide donor, sodium nitroprusside (SNP, 50 mM) was applied to the superfusate to examine the loading status of endothelial cells in the vessel wall. MetaFluor software (Universal Imaging) was used for image analysis. Quantitative analysis was conducted using selected regions of interest (ROIs) along the vessel wall to measure the FI at the individual endothelial cell level. Each ROI covers the area of one endothelium, as indicated by the fluorescence outline upon the addition of SNP. The maximum stimulated FI_{DAF} (at plateau level) was compared with the control FI_{DAF} measured in the same ROI and expressed as $(FI/FI_0) \times 100$, in which FI is the FI_{DAF} after stimulation and FI_0 is the control FI_{DAF}. The criterion for a responsive cell was a significant increase in FI_{DAF} relative to the control in each ROI. The increase in FI_{DAF} in each vessel was the mean of the FI_{DAF} changes from all the responsive cells.

[4] NO production rate was derived from differential conversion of the cumulative FI of DAF-2. Details about the quantification of FI have been described.[4]

Solutions and reagents

Mammalian Ringer solution[5] was used for dissecting mesenteries, superfusing tissues, and preparing perfusion solutions. The high Ca^{2+} solution contains the same composition as that in normal mammalian Ringer solution except that $CaCl_2$ was increased from 2 mM to 10 mM. All perfusates used for control and test perfusion contained BSA (10 mg/ml). AP-CAV, the fusion peptide of caveolin-1 scaffolding domain (CAV, amino acids 82-101, DGIWKASFTTETVTKYWFYR) with AP, the Antennapedia internalization sequence from Drosophila Antennapedia homeodomain (amino acids 43-58, RQIKIWFQNRRMKWKK), AP-CAV-X, the fusion peptide of scrambled CAV (WGIDKAFFTTSTVTYKWFRY) with AP were custom synthesized by Tufts University with sequences identical to that published by Bucci et al..[6] DAF-2 DA, 1-*O*-alky1–2-acetyl-*sn*-glycero-3-phosphocholine (PAF), and sodium nitroprusside (SNP) were purchased from Sigma. Fura 2-AM was purchased from Molecular Probes. The stock solutions of DAF-2 DA (10 mM), fura 2-AM (10 mM) and each peptide (10 mM) were prepared with 100% DMSO. The final concentrations of DAF-2 DA (5 μM), fura 2-AM (10 μM) were achieved by 1:1,000 dilutions of the stock with albumin-Ringer solution, respectively. The final concentration of DMSO in AP-CAV and AP-CAVX peptide (1 μM) solutions was 0.01% (v/v). PAF was initially dissolved in 95% ethyl alcohol (5 mM) and further diluted to a final concentration of 10 nM with albumin-Ringer solution. All perfusates that contained the test agent were freshly prepared before each cannulation.

Reference

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